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Commentary

Fleeting opportunities

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Perhaps the major challenge in any experimental science is to identify and characterize species with fleeting lifetimes. Such species are often intermediates in a complex pathway, either biological or chemical. Numerous methods have been developed to achieve this goal, depending on the field of interest and the nature of the species in question. In biological studies of metabolism, a traditional method is to block a pathway and allow a metabolite to build up. In chemistry, one can reduce the temperature of a reaction and thereby trap an intermediate. In biochemistry, the elegant tools of stop-flow and fast spectroscopy allow kinetic characterization of species with lifetimes as short as nanoseconds or less. Femtosecond spectroscopy has permitted direct observation of transition-state processes in very simple molecules. These methods give fleeting glimpses of small molecules or small parts of large molecules.

In structural biology, the task is more formidable: to obtain structural information about short-lived species whose molecular weights can range into the hundreds of thousands at a resolution comparable to that achievable for the corresponding stable species. In the case of proteins and nucleic acids, this means atomic resolution. Since there is no such thing as a free lunch, especially where structure is concerned, obtaining atomic resolution requires large amounts of data to be collected. And that takes time.

It would seem, therefore, that atomic resolution and short lifetimes are incompatible. This conundrum has yielded somewhat in recent years to a new and exciting technology: time-resolved protein crystallography (1). The development of high-intensity x-ray sources from synchrotron radiation (2), combined with the use of polychromatic and fast monochromatic data-collection techniques (1), has allowed atomic-resolution x-ray diffraction data to be collected on a time scale of minutes to seconds. For very slow enzyme-catalyzed reactions, where a poor substrate can be diffused into a protein crystal in times shorter than that required for the subsequent chemistry, a set of high-resolution "snapshots" can be obtained by rapid monochromatic data collection (3). More often, diffusion into the crystal lattice is too slow relative to the lifetime of the species of interest, and photochemical triggering of "caged" precursors must be used to initiate the reaction throughout the entire crystal at a known instant in time, followed by very rapid data collection by the Laue method (4).

In the Laue method, a stationary crystal is bathed in a polychromatic x-ray beam. If the wavelength range in the beam is broad enough, and if the crystal belongs to a high-symmetry space group so that its unique x-ray data are packed into a small region of space, and if the x-ray beam is of very high intensity (i.e., synchtrotron radiation), then a single Laue photograph, which can be recorded in a fraction of a second, will contain a high percentage of the x-ray data needed for a high-resolution structure (5).

The Laue method is simple to carry out, but there are a number of considerations that can restrict its application. First, the crystalline specimen must be of high quality; even a small amount of disorder, which could be tolerated in monochromatic data collection, will prevent analysis of a Laue photograph. Second, the geometry of Laue diffraction causes the data observable on a single Laue photograph to be systematically incomplete. Overcoming this incompleteness may require a number of additional exposures, which negates the advantage of fast temporal resolution afforded by single-exposure data collection. Finally, it is often impossible to obtain a series of time-lapse photographs from a single specimen, since the high-intensity white beam is very destructive to proteins.

Despite these limitations, the Laue method has been used successfully in a number of time-resolved and other crystallographic studies. Small-molecule inhibitors and heavy-metal ions bound to large proteins have been located in electron-density maps obtained with Laue data (6, 7). An enzyme–substrate complex has been observed at high resolution by photolysis of a caged precursor followed by rapid Laue data collection (8). And the photochemical release of a covalent inhibitor from the active site of an enzyme, followed by the binding of a suicide substrate, has been studied by means of a series of Laue data sets taken over the time period of the reaction (9).

But the benefits of rapid data collection are not limited to the study of unstable complexes. They also have applicability to situations where one needs to collect a large amount of data from a small number of crystals.

Wood *et al.* (10) determined and refined the structure of the mineral berlinite with Laue data in 1983, and Harding and colleagues (11) have determined several small-molecule structures by using Laue diffraction data. A comparison of the crystal structures of a small molecule determined from both monochromatic and Laue data showed that Laue data sets can be comparable in quality to those obtained conventionally (12). The first protein structure determination using Laue diffraction data was that of turkey egg-white lysozyme by Howell *et al.* (13), who used the method of molecular replacement to solve the phase problem. A single 2-sec exposure produced enough data (67% of theoretical) to solve and refine the structure at 2.5-Å resolution.

All of these Laue structure determinations were done to test the method. In this issue of the *Proceedings*, a protein structure determination using Laue data is described that was impossible to do by any other means. Vellieux *et al.* (14) wished to use the three-dimensional structure of glycosomal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from the sleeping-sickness parasite *Trypanosoma brucei* as the basis for the rational design of specific inhibitors of this essential parasite protein. Unfortunately, they could obtain only milligrams of purified protein, enough to grow only a small number of small protein crystals. Imagine the dilemma this shortage of specimens presents to the crystallographer: how does one collect data from a limited number of crystals, when one does not know without actually collecting the data whether radiation damage will allow a sufficient number of reflections to be obtained before one runs out of crystals? In conventional methods of data collection one can work progressively out towards high resolution and stop when decay is appreciable, but for drug-design purposes high resolution is imperative. A preliminary assessment of radiation lifetime was made by Vellieux *et al.*, and the results were discouraging: although the trypanosomal GAPDH crystals initially scattered to 2.3-Å resolution,

after a short time in the x-ray beam only data to 4-Å resolution remained.

The Laue method provided a solution to these problems. By using a total of two crystals and 12.5 sec of total x-ray exposure, a set of data was collected that was 37% complete to a resolution of 3.2 Å. Despite the partial nature of these data, Vellieux *et al.* were able to use them to solve the structure of the trypanosomal GAPDH by the method of molecular replacement. Using the atomic coordinates of a bacterial GAPDH as a model, they were able to locate the positions of the six subunits in the crystallographic asymmetric unit. By employing additional Laue data from one more crystal and by exploiting the sixfold redundancy of information in the structure, they refined the trypanosomal GAPDH structure to a quality comparable to that of structures obtained conventionally. Comparison of the parasite enzyme with that from human sources reveals significant structural differences in the active-site region, which can be used as the basis for the design of selective compounds.

As structural biology tackles increasingly large and complex problems, the likelihood that only a small number of small crystals will be available for a structure determination increases. Indeed, such problems have arisen in the past and have led to the abandonment of a number of important projects. Now, methods derived from time-resolved crystallography can be used to grapple with such pathological cases. Although the systematic incompleteness of Laue data has thus far prevented the use of isomorphous replacement methods for phase determination (15), molecular replacement has now succeeded in two cases (13, 14) and is clearly compatible with the restrictions of the Laue method. As the structural data base increases, it becomes more likely that any given unknown structure will have a homolog whose structure is known. In such cases, molecular replacement is in fact the phasing method of choice. So, if you have only small quantities of an important macromolecule, do not despair. If you can obtain crystals, you may still be able to obtain a structure. But it is well to remember in such cases, even more than in cases where unconventional methods are not needed, that "Life is short, the art is long, opportunity fleeting, experience treacherous, judgement difficult" (Hippocrates, *Aphorisms*, Vol. I, page 1).

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